Impaired Trafficking of the Desmoplakins in Cultured Darier's Disease Keratinocytes

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Darier's disease is an autosomal dominantly inherited skin disorder characterized by loss of adhesion between epidermal cells, breakdown of desmosome–keratin filaments, and abnormal keratinization. *ATP2A2* has been identified as the causative gene of Darier's disease. This gene encodes the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) isoform 2 pump, which transports Ca^{2+} from the cytosol into the endoplasmic reticulum lumen to maintain a low cytosolic Ca^{2+} concentration. Using indirect immunofluorescence and biochemical analysis, we investigated the distribution of key desmosomal proteins in normal human and Darier's disease keratinocytes under various calcium conditions. We show that inhibition of SERCA by thapsigargin in normal human keratinocytes impairs the trafficking of the desmoplakins, desmoglein, and desmocollin to the cell surface; these proteins show a diffuse cytoplasmic distribution and, together with plakoglobin, form detergent-insoluble aggregates. In Darier's disease keratinocytes, only the trafficking of desmoplakin is significantly inhibited; in these cells, desmoplakin forms insoluble aggregates when extracted with mild detergent. In contrast, the transmembrane proteins desmoglein and desmocollin are efficiently transported to the cell surface. These proteins, along with plakoglobin, remain equally distributed between detergent-soluble and -insoluble fractions. We also demonstrate an interaction between SERCA2 and desmoplakin during differentiation. Our results provide further insights into the critical role of calcium ATPases in maintaining epidermal integrity. ***Key words: SERCAZ/Darier's disease/desmosomes. J Invest Dermatol 121:1349–1355, 2003***

Darier's disease (DD) is an autosomal dominant inherited skin disease characterized by loss of adhesion between epidermal cells (acantholysis) and abnormal keratinization (Burge and Wilkinson, 1992). Desmosome–keratin filament complexes are disrupted in acantholytic cells (Mann and Haye, 1970; Ishibashi *et al*, 1984), and antibody studies suggest that most desmosomal proteins are diffusely distributed in the cytoplasm (Burge and Garrod, 1991; Burge and Schomberg, 1992; Hashimoto *et al*, 1995; Hakuno *et al*, 2000). We identified *ATP2A2*, which encodes the sarco(endo) plasmic reticulum Ca^{2+}-ATPase (SERCA) 2 as the defective gene in DD (Sakuntabhai *et al*, 1999).

SERCA is a membrane bound Ca^{2+}-activated ATPase that transports calcium ions from the cytosol into the endoplasmic reticulum (ER) lumen to maintain a low cytosolic Ca^{2+} concentration (MacLennan *et al*, 1985). Most calcium in cells is sequestered in the lumen of the ER and is subject to release in response to many external stimuli mediated via inositol triphosphate and ryanodine receptors (Clapham, 1995). The release of ER calcium increases cytosolic calcium concentrations and stimulates calcium-dependent enzymes, such as kinases and phosphatases, that can modulate regulatory processes in the cytosol. Such processes include gene expression, the cell cycle, and apoptosis (Clapham, 1995). Changes in the ER Ca^{2+} concentration also affect many functions of the ER, including protein synthesis, protein folding involving chaperones, and protein secretion (Corbett and Michalak, 2000).

Desmosomes are symmetrical intercellular junctions found primarily in epithelial tissues. They are composed of members from three protein families: the cadherins, the armadillos, and the plakins. The transmembrane cadherin family consists of desmogleins (Dsg) and desmocollins (Dsc), which interact with members of the armadillo family including plakoglobin (PG) and the plakophilins, which in turn bind to desmoplakin (Dp), a major plaque protein (Green and Gaudry, 2000). The assembly of desmosomes can be studied *in vitro* using a Ca^{2+} switch system (Hennings *et al*, 1980). Under low Ca^{2+} conditions, desmosomes are not assembled but constituent proteins continue to be synthesized (Duden and Franke, 1988), and following a Ca^{2+} shift to physiologic levels, desmosomal proteins are gradually targeted to the basolateral membrane where they form mature desmosomes (Pillai *et al*, 1988).

The pathogenesis of abnormality in DD cells regarding desmosome function remains unclear. The finding that mutations in SERCA2 pump cause the disease supports the importance of intracellular calcium homeostasis for desmosomal adhesion. It is possible that SERCA2 may influence expression, recruitment, sorting, or association of desmosomal components. In this study, we have investigated the mechanisms by which SERCA regulates

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Abbreviations: DD, Darier's disease; Dsc, desmocollins; Dp, desmoplakins; Dsg, desmogleins; ER, endoplasmic reticulum; MDCK, Madin-Darby canine kidney; NHK, normal human keratinocyte; PG, plakoglobin; SERCA, sarco(endo)plasmic reticulum Ca^{2+}-ATPase.

the distribution of key desmosomal proteins in normal human and DD keratinocytes. In addition, we demonstrate a novel protein–protein interaction involved in the differentiation of keratinocytes.

MATERIALS AND METHODS

Reagents and cell culture All reagents and chemicals were obtained from Sigma (Poole, UK) unless otherwise stated. Culture media were obtained from Invitrogen (Paisley, UK).

The Central Oxfordshire Research Ethic Committee approved the study and all patients gave informed consent. Biopsies were taken from the unaffected abdominal skin of two unrelated DD patients with known heterozygous mutations in SERCA2. One patient was heterozygous for a missense mutation and the other for a nonsense mutation, which predict misfolding and no protein production, respectively. Control biopsies of normal skin were obtained from four age-matched controls during routine surgical procedures.

The biopsies were explanted and primary keratinocytes were isolated and grown as previously described (Rheinwald and Green, 1975; Simon and Green, 1985). Keratinocytes were grown in keratinocyte-serum-free low-calcium medium (<0.1 mM) for one passage to reduce 3T3 contamination, before further experiments. Keratinocytes were seeded in keratinocyte-serum free-low calcium medium overnight. Cells at 70% to 80% confluence were then incubated in the absence or presence of 100 nM thapsigargin or 5 μg per mL tunicamycin for 30 min before induce differentiation in normal-calcium medium (1.2 mM) for 4 h.

Antibodies Mouse monoclonal antibodies recognizing Dsg1, Dp1, and Dp2; rabbit anti-Dsc3; and guinea pig anti-Dsc2 were provided by D. Garrod (University of Manchester, Manchester, UK). Mouse monoclonal anti-PG antibody was purchased from Cymbus Bioscience (Southampton, UK), goat polyclonal anti-SERCA2 antibody was from Santa Cruz Biotechnology (Insight Biotechnology, Middlesex, UK), and rabbit polyclonal anti-calnexin antibody was obtained from Calbiochem (CN Biosciences, Nottingham, UK).

Immunofluorescence For immunofluorescence analysis, keratinocytes grown on eight-well chamber slides were processed as previously described (Cobbold *et al*, 1996). Briefly, cells were fixed in $-20^{\circ}C$ methanol and blocked in 0.2% bovine serum albumin in phosphate-buffered saline containing 0.1% Triton X-100. Bound mouse anti-Dp and anti-Dsg1 and rabbit anti-Dsc3 were detected using fluorescein isothiocyanate conjugated with corresponding secondary antibodies. Cells were viewed using a Nikon Optiphat microscope and a $60\times$ oil objective operating on a Bio-Rad 1024 confocal system. Images were collected using the Bio-Rad Lasersharp software for viewing, analysis, and quantification. To avoid bleed-through from different channels, sequential capture of each image was performed on double-labeled samples. Fluorescence intensity was estimated as an average pixel intensity of digitized images measured from equivalent areas containing the plasma membrane regions from 30 cells from five different fields using laser scanning confocal microscope analysis software (Bio-Rad Laboratories, Hercules, CA). Each experiment was performed a minimum of three times.

Protein extraction and immunoblotting Keratinocytes were washed once with phosphate-buffered saline and lyzed in 1% Triton X-100 immunoprecipitation buffer (10 mM Tris (pH 7.8), 0.15 M NaCl, 10 mM iodoacetamide, 1 mM EDTA, 1 mM phenylmethysulfonyl fluoride, and 1 μg each of leupeptin, pepstatin, chymostatin, and antipain (Boehringer Mannheim, Lewes, UK) per mL) for 20 min at $4^{\circ}C$. Cell extracts were separated by centrifugation at $10{,}000\times g$ for 30 min, and the insoluble residue was resuspended in lysis buffer. Cell lysates or equivalent aliquots of each fraction were mixed with Laemmli sample buffer, boiled for 5 min, resolved by 5% to 7.5% SDS–PAGE and transferred onto polyvinylidene difluoride membranes (Millopore, Herts, UK). Blots were probed with primary antibody and anti-mouse or anti-rabbit secondary antibodies linked to peroxidase (Amersham Pharmacia Biotech, Buckinghamshire, UK) followed by enhanced chemiluminescent blotting (Amersham).

Immunoprecipitation Cells were washed once in phosphate-buffered saline, lyzed in 1% Triton X-100 immunoprecipitation buffer, and precleared with protein G preclear (Amersham) for 20 min at $4^{\circ}C$. Precleared total lysates were incubated with 1 μg of anti-SERCA2 or anti-Dp antibody with protein G–Sepharose (Amersham) at $4^{\circ}C$ overnight. Immunocomplexes bound to Sepharose beads were washed with 1% Triton X-100 immunoprecipitation buffer, resuspended in Laemmli sample buffer, and boiled before loading on 7.5% SDS–PAGE. Immunoblotting was then performed as above.

Metabolic labeling Cells were metabolically labeled with 1.85 MBq per mL [^{35}S]methionine and cysteine overnight and then chased in high-calcium medium for 4 h. Cells were lyzed, and processed for immunoprecipitation as described above. Cell lysates were analyzed by 7.5% SDS–PAGE and fluorography.

RESULTS

Thapsigargin inhibits the trafficking of desmosomal proteins to the cell surface The calcium switch is one of the most widely used systems to study desmosome assembly (Hennings *et al*, 1980). Under low-calcium conditions, desmosomal assembly does not occur at the cell surface, but proteins continue to be synthesized. The Dsg and Dsc are transmembrane proteins synthesized on the ER, whereas the plaque proteins, such as PG and the Dp, are synthesized on free ribosomes in the cytoplasm. On shifting to normal-calcium conditions, desmosomal proteins are gradually targeted to the cell surface (Duden and Franke, 1988; Pillai *et al*, 1988). In this study, we used this calcium-shift method in primary keratinocytes to analyze changes in the cellular distribution of the desmosomal proteins Dp, Dsg, and Dsc in response to changes in calcium concentration.

Under low-calcium conditions Dp, Dsg, and Dsc were diffusely distributed, and very little protein was detected at the cell surface (**Fig 1*A,D,G***). Nevertheless, when cells were incubated in normal-calcium medium to induce cell differentiation, these desmosomal proteins showed substantial redistribution to the plasma membrane (**Fig 1*B,E,H***). These are similar to results observed in the Madin-Darby canine kidney (MDCK) cell line (Penn *et al*, 1987a, b; Pasdar and Nelson, 1988a, 1989).

Thapsigargin, a highly specific and potent inhibitor of the SERCA pumps, selectively depletes calcium from the ER lumen (Thastrup *et al*, 1989). Previous reports in MDCK cells have shown that thapsigargin treatment inhibits the trafficking of Dp and the tight junction protein, ZO-1, to the plasma membrane. Both proteins were detected in a detergent-soluble pool and did not enter detergent-insoluble fractions (Stuart *et al*, 1996).

To further analyze the effects of SERCA dysfunction on desmosome assembly in primary keratinocytes, cells were treated with thapsigargin before differentiation. None of the desmosomal proteins were expressed strongly at the plasma membrane after thapsigargin treatment (**Fig 1*C,F,I***). Digital-based microscopy techniques allow us to quantify the proportion of each desmosomal protein present at the plasma membrane in a statistically significant manner (Cobbold *et al*, 2002, 2003). A quantitative assessment of plasma membrane Dp, Dsg, and Dsc levels is shown in **Fig 1*J***. These data show that plasma membrane levels of Dp and Dsg increased by approximately threefold after differentiation, whereas Dsc levels were sixfold higher. In contrast, levels of cell surface staining in the presence of thapsigargin were similar to those observed in low-calcium medium. These data suggest that inhibition of SERCA with thapsigargin inhibits the efficient trafficking of Dp, Dsg, and Dsc to the cell surface in normal human keratinocytes (NHK).

Dsg and Dsc, but not the Dp, colocalize with the ER membrane protein, calnexin To determine the cellular distribution of these desmosomal proteins under low-calcium conditions, cells were double stained with antibodies to subcellular membrane compartments. Dsg and Dsc displayed substantial codistribution with the endoplasmic marker protein calnexin (**Fig 2*D-I***). This was as expected because these two integral membrane proteins are synthesized on ER-bound ribosomes. In contrast, the cytosolically synthesized plaque protein, Dp, showed little overlap with calnexin (**Fig 2*A–C***),

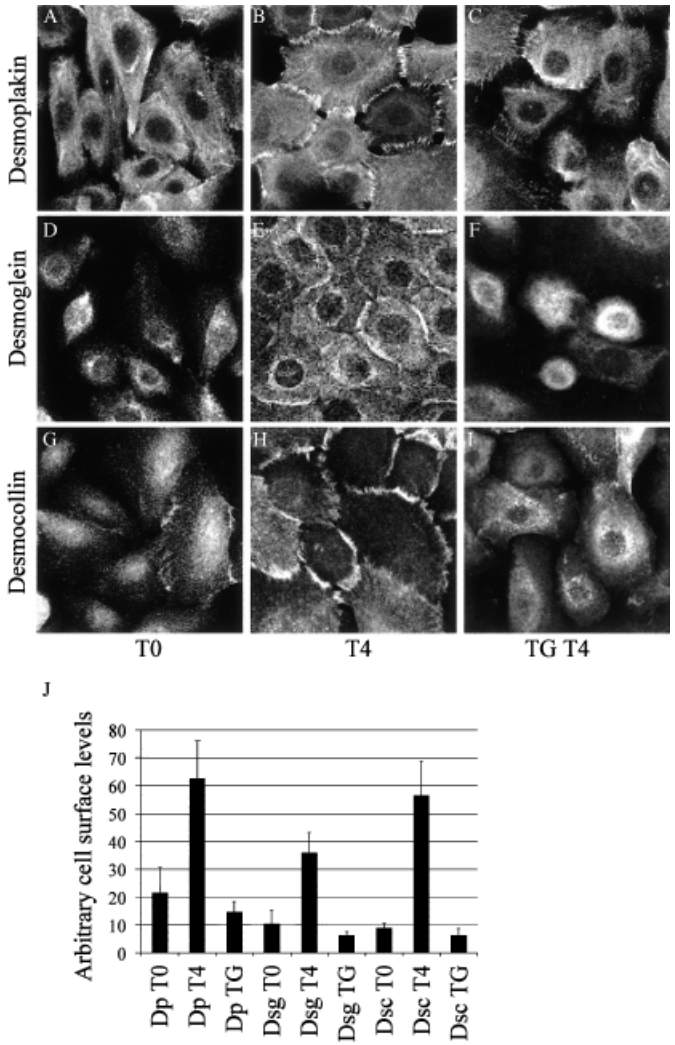

Figure 1. Thapsigargin inhibits the trafficking of desmosomal proteins to the cell surface. NHK cells were incubated in low-calcium medium (*A,D,G*) or incubated in the presence (*C,F,I*) or absence of thapsigargin (*B,E,H*) for 30 min. Medium was removed, and cells were incubated in normal-calcium medium for an additional 4 h to induce cell differentiation (*B,C,E,F,H,I*). Cells were fixed in methanol and processed for indirect immunofluorescence and confocal microscopy. Bound mouse anti-Dp, anti-Dsg, and guinea pig anti-Dsc were detected using fluorescein isothiocyanate-conjugated antibody for the corresponding species. (*J*) Histogram showing quantification of the relative levels of plasma membrane protein in response to different conditions.

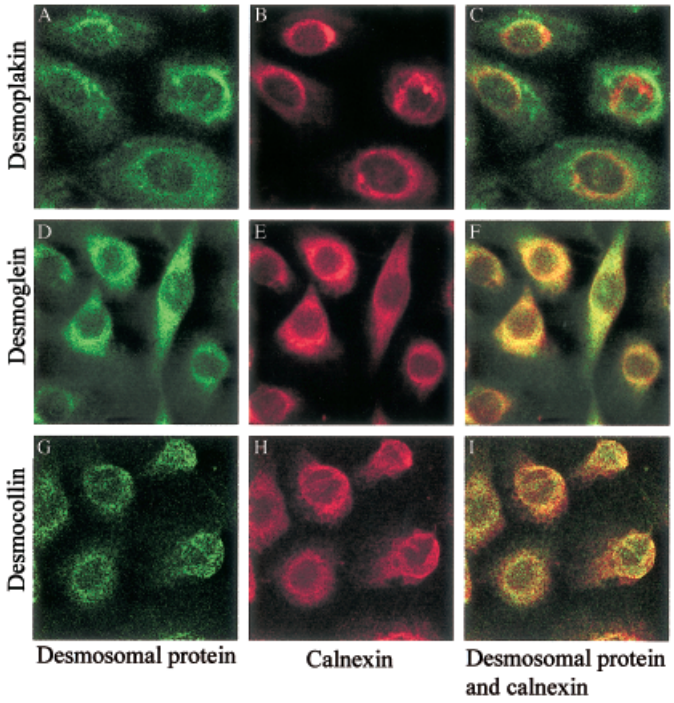

Figure 2. Desmosomal proteins colocalize with the ER membrane protein calnexin. NHK cells incubated under low-calcium conditions were fixed in methanol and processed for indirect immunofluorescence and confocal microscopy. Bound mouse anti-Dp, anti-Dsg, and guinea pig anti-Dsc (*green*) and anti-rabbit calnexin (*red*) were detected using fluorescein isothiocyanate-conjugated anti-mouse/guinea pig and Texas Red-conjugated anti-rabbit secondary antibodies.

suggesting that Dp does not bind ER membranes before cell differentiation.

Desmosomal proteins form detergent-insoluble aggregates and are correctly glycosylated in the presence of thapsigargin Dp and PG are plaque proteins synthesized in the cytosol, whereas Dsg and Dsc are transmembrane proteins synthesized on the ER membrane. In an attempt to further characterize the cellular distribution of these proteins under various calcium conditions, primary keratinocytes were separated into detergent-soluble and detergent-insoluble (aggregate) fractions. Under both low (T0) and normal (T4) calcium conditions, 40% to 60% of each protein was distributed in the detergent-soluble fraction, with the remainder observed in the detergent-insoluble fraction (**Fig 3**).

A previous report in MDCK cells showed that thapsigargin treatment increased the detergent solubility of Dp and inhibited its transport to the cell surface (Stuart *et al*, 1996). We also showed that the trafficking of the desmosomal proteins, including Dp, was inhibited in primary keratinocytes (**Fig 1**). We therefore tested the effects of thapsigargin on the solubility of these key desmosomal proteins in primary keratinocytes. Interestingly, our data show that the majority of all four desmosomal proteins were detected in the detergent-insoluble fraction when cells were incubated with thapsigargin before differentiation (TG); very little protein was observed in the detergent-soluble fractions (**Fig 3**).

The observation that thapsigargin treatment caused the desmosomal proteins to form insoluble aggregates, and also prevented the transport of desmosomal proteins from the ER, led to the possibility that the proteins may not be correctly glycosylated and are therefore misfolded. Experiments using thapsigargin and tunicamycin, a chemical that inhibits protein glycosylation, showed that the nonglycosylated proteins, Dp and PG, did not differ in size in the presence of either tunicamycin or thapsigargin compared to the control (undifferentiated or differentiated; data not shown). Dp and PG were migrating at their correct size. Nevertheless, after tunicamycin treatment, two protein bands were detected for the glycosylated proteins, Dsg and Dsc (not shown). The higher band corresponded to the glycosylated protein, and the lower band to the unglycosylated form. The glycosylated protein is most likely synthesized before tunicamycin addition, whereas the unglycosylated form is protein synthesized after addition of tunicamycin. In contrast, when cells were incubated with thapsigargin, only the higher, correctly glycosylated form of Dsg and Dsc proteins were detected (data not shown). Preincubation with thapsigargin, unlike tunicamycin, does not affect glycosylation.

The trafficking of Dp is inhibited in DD keratinocytes In the experiments described above we showed that inhibiting

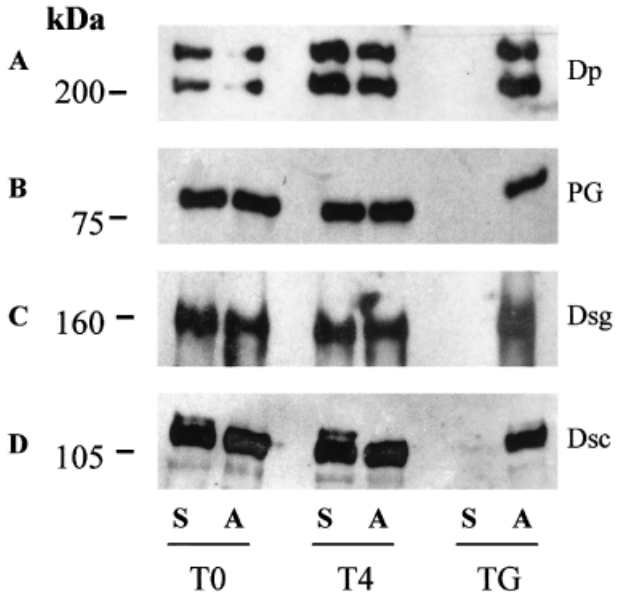

Figure 3. Desmosomal proteins form insoluble aggregates in the presence of thapsigargin. NHK cells were incubated in low-calcium medium or incubated in the absence or presence of thapsigargin for 30 min, medium was removed, and cells were incubated in normal-calcium medium for an additional 4 h. Cells were lyzed in mild detergent, and lysates were centrifuged at 10,000 g for 30 min to recover a pellet of insoluble protein aggregates (*A*) and a supernatant containing cytosol and solubilized membrane proteins (*S*). Samples were analyzed by SDS-PAGE and western blot using antibodies to Dp (*A*), PG (*B*), Dsg (*C*), and Dsc (*D*).

SERCA function with thapsigargin in primary keratinocytes causes desmosomal proteins to inefficiently traffic to the cell surface and form detergent-insoluble aggregates. To gain insight into the molecular mechanisms that lead to DD, the distribution of desmosomal proteins was explored in primary keratinocytes from two DD patients. DD keratinocytes were incubated in low- and normal-calcium medium, and the localization of the desmosomal proteins was analyzed by immunofluorescence and confocal microscopy. In low-calcium medium, all desmosomal proteins showed a diffuse staining pattern, and very little protein was detected at the cell surface (**Fig 4*A,C,E***). The results observed were similar to those observed in NHK control cells (**Fig 1**). When DD cells were incubated in normal-calcium medium to induce cell differentiation, Dsg (**Fig 4*D***) and Dsc (**Fig 4*F***) showed substantial redistribution to the plasma membrane. Quantitative analysis (**Fig 4*G***) shows that plasma membrane Dsg and Dsc levels increased 3- and 4-fold, respectively. In contrast, Dp trafficking to the plasma membrane was significantly inhibited in DD cells (**Fig 4*B***) compared to NHK (**Fig 1*B***). A quantitative assessment showed that cell surface levels were only increased by 1.5-fold (**Fig 4*G***) compared to 3-fold in NHK cells (**Fig 1*J***). These data suggest that the trafficking of all three desmosomal proteins studied was inhibited in DD cells; nevertheless, the most marked effect was on Dp. The same results were obtained using cells from both DD patients (see Materials and Methods).

Dp forms detergent-insoluble aggregates in DD keratinocytes The trafficking of desmosomal proteins to the cell surface was less efficient in DD cells than NHK cells. We have demonstrated that inhibition of the SERCA calcium pump with thapsigargin caused abnormal distribution of Dp, Dsg, Dsc, and PG (**Fig 3**). It is possible that the reduced trafficking of desmosomal proteins in DD cells may be due to inappropriate aggregation and cellular distribution. To test this possibility, DD cells incubated in low- or normal-calcium medium were

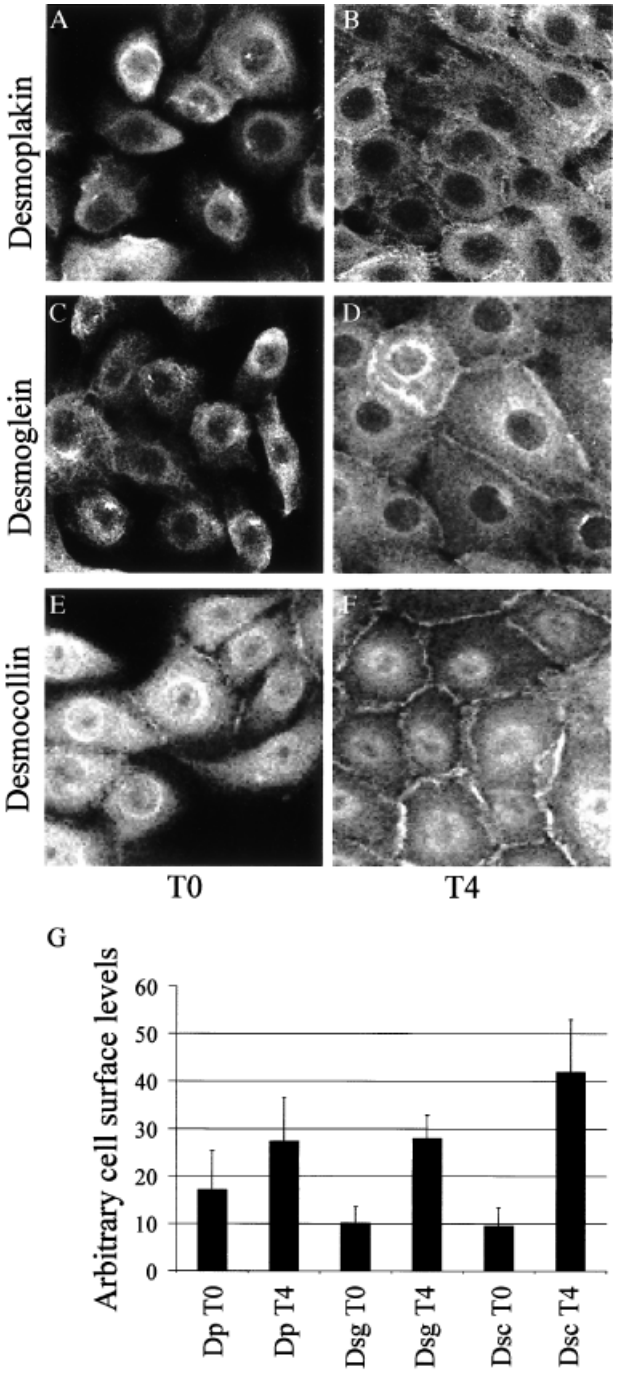

Figure 4. The trafficking of Dp is inhibited in DD keratinocytes. DD cells were incubated in low-calcium medium (*A,C,E*) or incubated for 4 h in normal-calcium medium to induce cell differentiation (*B,D,F*). Cells were fixed in methanol and processed for indirect immunofluorescence and confocal microscopy. Bound mouse anti-Dp, anti-Dsg and guinea pig anti-Dsc were detected using fluorescein isothiocyanate-conjugated antibody for the corresponding species. (*G*) Histogram showing quantification of the relative levels of plasma membrane protein of DD keratinocytes in comparison with NHK in response to different calcium conditions.

extracted with mild detergent and separated into detergent-soluble and detergent-insoluble fractions. In uninduced and induced DD cells, 40% to 60% of the total of Dsg (**Fig 5*B***), Dsc (**Fig 5*C***), and PG (**Fig 5*D***) were recovered in the detergent-soluble fraction (S). This was a similar result to that

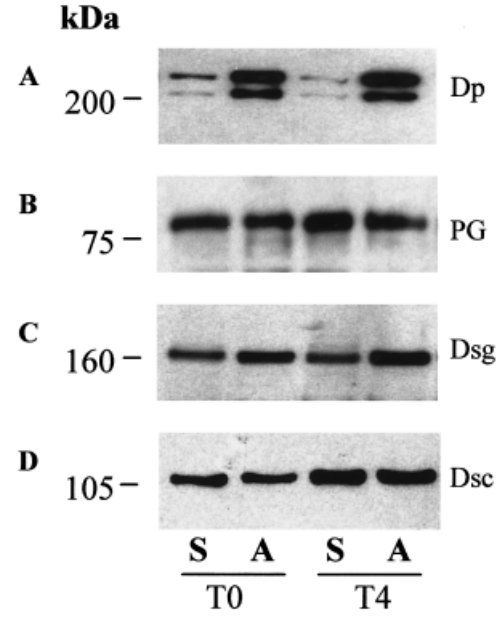

Figure 5. Dp forms detergent-insoluble aggregates in DD keratinocytes. DD cells were incubated in low-calcium medium or incubated for 4 h in normal-calcium medium to induce cell differentiation. Cells were lyzed in mild detergent, and lysates were centrifuged at 10,000 g for 30 min to recover a pellet of insoluble protein aggregates (*A*) and a supernatant containing cytosol and solubilized membrane proteins (*S*). Samples were analyzed by SDS-PAGE and western blot using antibodies to Dp (*A*), PG (*B*), Dsg (*C*), and Dsc (*D*).

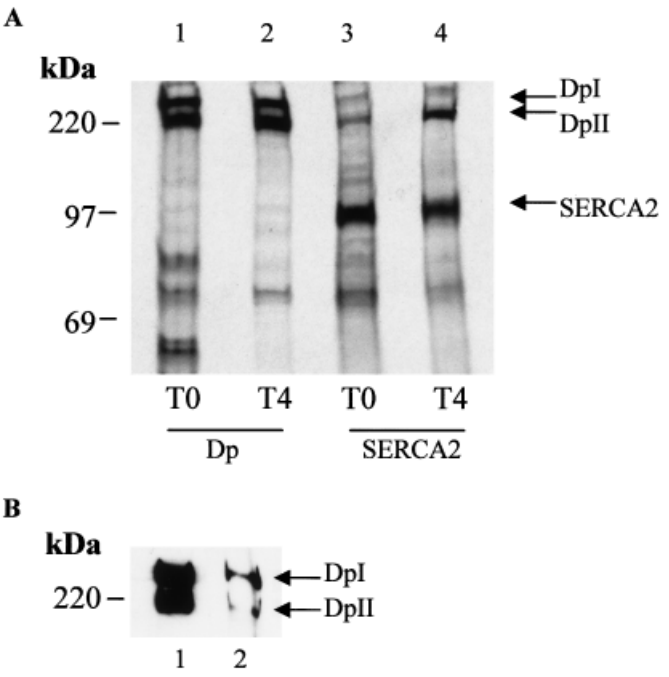

Figure 6. Dp associates with SERCA2 during differentiation. (*A*) NHK cells grown in low-calcium medium were pulse-labeled with [^{35}S]methionine and cysteine and then chased in a normal-calcium medium for 4 h. Cells were lyzed and immunoprecipitated with the indicated antibody. (*B*) NHK cells were incubated in normal-calcium medium for 4 h to induce cell differentiation. Cells were lyzed and immunoprecipitated with anti-SERCA2 antibody. SERCA2 immunoprecipitates were analyzed by SDS-PAGE and western blot using an anti-Dp antibody (*lane 2*). *Lane 1,* Western blot of total cell extract.

observed in NHK cells (**Fig 3**). In the case of Dp (**Fig 5*A***), however, the majority of the protein detected in cells pelleted with the detergent-insoluble fraction (A). Quantitative analysis showed that greater than 90% of Dp formed aggregates before and after induction with calcium. Similar Dp aggregates were obtained when NHK cells were induced to differentiate in the presence of thapsigargin (**Fig 1*C***). These data suggest that inhibition of SERCA2 function in DD keratinocytes has an adverse effect on the conformation, cellular distribution, and trafficking of Dp to the cell surface, whereas there is little effect on Dsg, Dsc, and PG.

Dp associates with SERCA2 during calcium-induced differentiation In this report, we have shown that Dp did not efficiently traffic to the cell surface and formed detergent-insoluble aggregates in DD cells. We also demonstrated that Dp does not colocalize to the ER under low-calcium conditions, whereas Dsc and Dsg remain in this membrane compartment. We therefore hypothesized that SERCA2 may mediate the assembly of desmosomal proteins, particularly Dp, by binding Dp in a calcium-dependent fashion and effecting its release to a desmosomal complex at the correct time and in the correct calcium conditions. To test for a possible interaction between SERCA2 and Dp, coimmunoprecipitation studies were undertaken.

NHK cells grown in low-calcium medium were pulse-labeled with [^{35}S]methionine and cysteine and then chased in normal-calcium medium for 4 h. Cells were lyzed and immunoprecipitated with either anti-Dp or anti-SERCA2 antibodies. **Figure 6*A*** shows detection of DpI and DpII after immunoprecipitation with anti-Dp in low- (*lane 1*) and normal- (*lane 2*) calcium medium, respectively. When cells were immunoprecipitated with anti-SERCA2 antibody (*lanes 3,4*), the SERCA protein was observed at 110 kDa. Interestingly, two protein bands were also detected migrating at approximately 250 and 215 kDa, the predicted sizes of DpI and DpII, respectively. Quantification revealed a threefold increase in the levels of these proteins on SERCA immunoprecipitates after differentiation. We did not detect SERCA2 in Dp immunoprecipitates (*lanes 1,2*). It is possible that the anti-Dp epitope shares the SERCA2-binding site, hence blocking SERCA2 binding during immunoprecipitation analysis.

To confirm an interaction between Dp and SERCA2, NHK cells were grown under normal-calcium conditions, lyzed, and immunoprecipitated with anti-SERCA2 antibody. SERCA2 immunoprecipitates were then analyzed by Western blot using an anti-Dp antibody (**Fig 6*B***). *Lane 1* shows the migration of the Dp proteins DpI and DpII recovered from total cell extracts. *Lane 2* shows that the SERCA2 antibody coprecipitated 250- and 215-kDa proteins (DpI and DpII) that were detected with the anti-Dp antibody. Together, these results implicate an interaction between SERCA2 and Dp in primary keratinocytes that increases in intensity when cells are switched to normal-calcium medium. This suggests that an interaction between Dp and SERCA2 may be required for efficient trafficking of Dp to the cell surface during differentiation.

DISCUSSION

We have investigated the link between the ER lumenal Ca^{2+} levels as regulated by the ER resident SERCA2 pump and the trafficking of key desmosomal proteins that regulate skin physiology, in primary human keratinocytes. We have extended these studies to keratinocytes from patients with DD to test whether SERCA2 function and desmosome assembly are dysregulated under such conditions.

Three different genes encode SERCA proteins. SERCA1 is exclusively expressed in fast-twitch skeletal muscle. SERCA2a is the cardiac/slow-twitch isoform of SERCA2, whereas SERCA2b,

with a C-terminal extension, is expressed in smooth muscle and nonmuscle tissue. SERCA3 is expressed in nonmuscle tissue, including endothelial, epithelial, lymphocytic cells, and platelets. Thapsigargin binds with high affinity to all SERCA proteins, inhibiting SERCA function and decreasing the Ca^{2+} concentration within the ER lumen (Bian *et al*, 1991; Ghosh *et al*, 1991; Sagara and Inesi, 1991). In normal keratinocytes, we showed that thapsigargin prevented efficient differentiation and transport of Dp, Dsg, and Dsc to the cell surface; these desmosomal components, along with PG, formed detergent-insoluble aggregates.

Previous studies in MDCK cells showed that Dp, Dsg, and Dsc are transferred from soluble to insoluble pools following a rise in calcium concentration (Pasdar and Nelson, 1988a, b, 1989; Penn *et al*, 1989). Using thapsigargin, the rate of Dp assembly at the cell surface was decreased and Dp was retained in a detergent-soluble pool (Stuart *et al*, 1996). In contrast, our results with primary keratinocytes showed that equal proportions of Dp, Dsg, Dsc, and PG were detected in detergent-soluble and -insoluble fractions under both low- and normal-calcium conditions. Significantly, all desmosomal proteins tested were found in the insoluble fraction after thapsigargin treatment. The different protein distributions observed in primary human keratinocytes (stratified epithelium) and MDCK cells (simple epithelial cell line) in the absence or presence of thapsigargin may reflect different mechanisms used for differentiation. Future studies will determine whether this is the case.

All isoforms of SERCA are inhibited by thapsigargin, whereas only SERCA2 function is impaired in DD cells. The trafficking of the transmembrane proteins, Dsg and Dsc, to the cell surface was slightly inhibited in DD cells compared to control cells, and the proteins were evenly distributed between detergent-soluble and -insoluble fractions. The plaque protein, PG, was also equally distributed between detergent-soluble and -insoluble fractions in DD cells. In contrast, we found that the other plaque protein, Dp, showed inhibited trafficking to the cell surface in DD keratinocytes, and that this protein was largely present in a detergent-insoluble fraction.

We also analyzed the effects of ionomycin, a calcium-specific ionophore (Liu and Hermann, 1978), on the solubility profile of desmosomal proteins and on their trafficking to the cell surface. Our results showed that all desmosomal proteins were efficiently transported to the cell surface, and the proteins were equally distributed between detergent-soluble and -insoluble fractions (J. Dhitavat, unpublished observations). These data suggest that the effects of SERCA2 disruption on Dp trafficking and solubility are due to defects in SERCA2, rather than a more general calcium imbalance between the cytoplasm and ER lumen.

Under low-calcium conditions, little Dp localized to the ER; nevertheless, upon shifting to normal calcium levels increased levels of the Dp–SERCA2 association were observed. What is the biologic significance of such an interaction? Desmosome assembly begins with the trafficking of Dsc to the cell surface, followed by Dsg and PG, with the final stages involving Dp (Burdett and Sullivan, 2002). It is possible that under low-calcium conditions, Dsc and Dsg are retained at the ER as partially folded or assembled complexes, perhaps in association with ER chaperones such as calnexin or calreticulin. Nevertheless, as calcium levels increase within the ER lumen, first Dsc, then Dsg together with PG, are transported to the cell surface where further maturation events take place. At the same time, it is likely that a calcium-induced interaction between SERCA2 and Dp occurs on the ER membrane. Our findings suggest that SERCA2 may have an accessory function, binding Dp, and allowing correctly folded/assembled Dp to traffic to the cell surface. Alternatively, an interaction between SERCA2 and Dp could regulate desmosomal assembly in a positive fashion by transiently binding one or more subunits and promoting functional assembly, possibly in association with other proteins. Upon release from SERCA2, the maturing Dp protein complex would then traffic to the cell surface. We did not detect interactions between SERCA2 and other desmosomal components and immunoprecipitation analysis with irrelevant antibodies did not show interactions with Dp or SERCA2 (J. Dhitavat, unpublished observations), further highlighting that this is a real and novel interaction.

In summary, these results increase our understanding of the molecular mechanisms underlying control of cell adhesion in normal skin and the impairment in adhesion between keratinocytes in DD. We have shown that mechanisms for Dp maturation and transport are different from those for Dsg, Dsc, and PG, and we provide evidence for a novel function of the SERCA2 calcium pump in mediating the assembly of the desmosomal complex.

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